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Disc Diffusion Sensitivity Testing of Candida Species

*Susan Verghese, **MC Sapna, ***P Padmaja and ****P Sudha*

Institute of Cardio-Vascular Diseases, Madras Medical Mission,
4-A, Dr. J. Jayalalitha Nagar, Mogappair, Chennai-600 050

SUMMARY

Fungal infections have become important in the modern era with many immunosuppressed patients and others who undergo many invasive procedures. Antifungal chemotherapy is selected primarily empirically. This study, a simple disc diffusion test for quick determination of susceptibility of Candida species to the four commonly used antifungal agents in our hospital was undertaken to determine the susceptibility or resistance of candida isolates. All the 155 strains were found to be sensitive to Fluconazole & Itraconazole respectively. C.tropicalis was the predominent species isolated (51.6%) followed by C.albicans (19.35%).

Key words : Candida species, disc diffusion sensitivity

INTRODUCTION

Fungal infections range from superficial infections to deep and systemic infections. These "systemic mycoses" occur primarily in patients with altered host defences usually due to underlying disease or due to various therapeutic interventions. Candida species is the most common organism causing fungal infections followed by Aspergillus species. Candida species is also the fourth most common nosocomial pathogen in most community and academic medical centers. The sensitivity of fungi are rarely tested on a routine basis in hospitals. However with reports of resistance to antifungal agents appearing in literature it was decided to carry out disc diffusion sensitivity tests on the candidal isolates encountered in our hospital.

MATERIALS AND METHODS

The culture medium used was Bacto Yeast Nitrogen Base as recommended by Casals (1979)[2] with slight modifications. It was prepared by taking

Bacto yeast Nitrogen Base (code 0392)	-	6.7 g
L. Asparagine	-	1.5 g
Glucose	-	10.0g
Distilled water [to make]	-	100 ml

After dissolution and sterilization by membrane filtration, a 10 x concentrate (Yeast Nitrogen Base conc. solution) remains. 1 litre of solid culture medium

*Associate Professor, Department of Microbiology; ** Junior Microbiologist *** Microbiologist-in-charge; **** Senior Microbiologist

for sensitivity tests was prepared by taking

HaH_2PO4	0.33g
K_2HPO4	0.92g
Agarose	11.0g
D. water to make	900 ml

The phosphate, agarose and water are melted by heating to 90°C to 100°C for 10 minutes. pH is adjusted to 7. Medium is sterilized by autoclaving at 121°C for 15 minutes. After cooling, 100 ml of Yeast Nitrogen Base concentrated sterile solution is added and poured into petriplates.

Addition of glucose to media greatly enhances the growth of yeasts but may cause lowering pH. Nevertheless the phosphate buffer should avoid a drastic lowering of pH during incubation.

Innoculum Seeding and Incubation

Suspension of fungus is made in 0.1 M phosphate buffer (0.33g NaH_2 PO4 + 0.92 K^2HPO4 in 1 litre of distilled water along with 0.1% Triton X 100) The isolates were subcultured onto fresh SDA plates. Five to six colonies were then suspended in Triton broth (5m) and turbidity adjusted to match MacFarland's 0.5. A sterile swab was dipped into this and streaked evenly on to the freshly prepared Yeast Nitrogen Agar base plate. Discs of Amphotericin B 100 mg/ml, traconazole 25mg & Nystatin 100 units were placed on it. incubation was done at 35°C. Reading were taken at 24 & 48 hours.

When testing with Fluconazole and Itraconazole a zone of partially inhibited colonies appears. This should be disregarded and the zone measured to colonies of normal size. The smaller colonies within the zone are not resistant mutants and their presence is due to mode of action of imidazole blocking the synthesis of sterols in fungi, but not to 100%

Interpretation of Degree of Sensitivity

SENSITIVE - Infection may be expected to respond to normal dosage of drug.

INTERMEDIATE ; Infection may be treated in some cases by using a high dosage of drug and may respond to concentration attainable in areas such as urinary tract.

RESISTANT - Drug cannot be used for treatment.

Table 1 : Criteria for following the Zone Diameters[3, 4]

	Nystatin 100 units	Fluconazole 20 mg Itraconazole 25 mg	Ampho.B 100 mg
Sensitive	> 12 mm	> 16 mm	> 10 mm
Intermediate	8-11 mm	10-15 mm	8-10 mm
Resistant	< 7 mm (up to disc.)	< 9 mm	< mm

Range of Antifungal Drugs Used

1. Nystatin - 100 unit/disc.
2. Amphotericin B - 100 mg/disc. Commercially available. Himedia discs have been used.
3. Fluconazole - disc of 20 mg/discs prepared from Fluconazole IV (Syscan) formulation which is commercially available.
4. Itraconazole - 40 mg of Itraconazole weighed out from commercially available capsule was dissolved by vortexing with glass beads with 2ml of Dimethyl formamide to get 4000 mg/ml stock. Further dilutions are made using distilled water to prepare 25mg/disc.

RESULTS

The results are as indicated in Table 2 to 4 and figure 1.

Table 2 : Distribution of Candida isolates according to drug scientivity

Total no. of isolates of Candida studied	-	155
Total no. of isolates Resistant to Nystatin	-	0
Amphotericin B	-	0
Fluconazole	-	5 (3.2%)
Itraconazole	-	3 (1.9%)

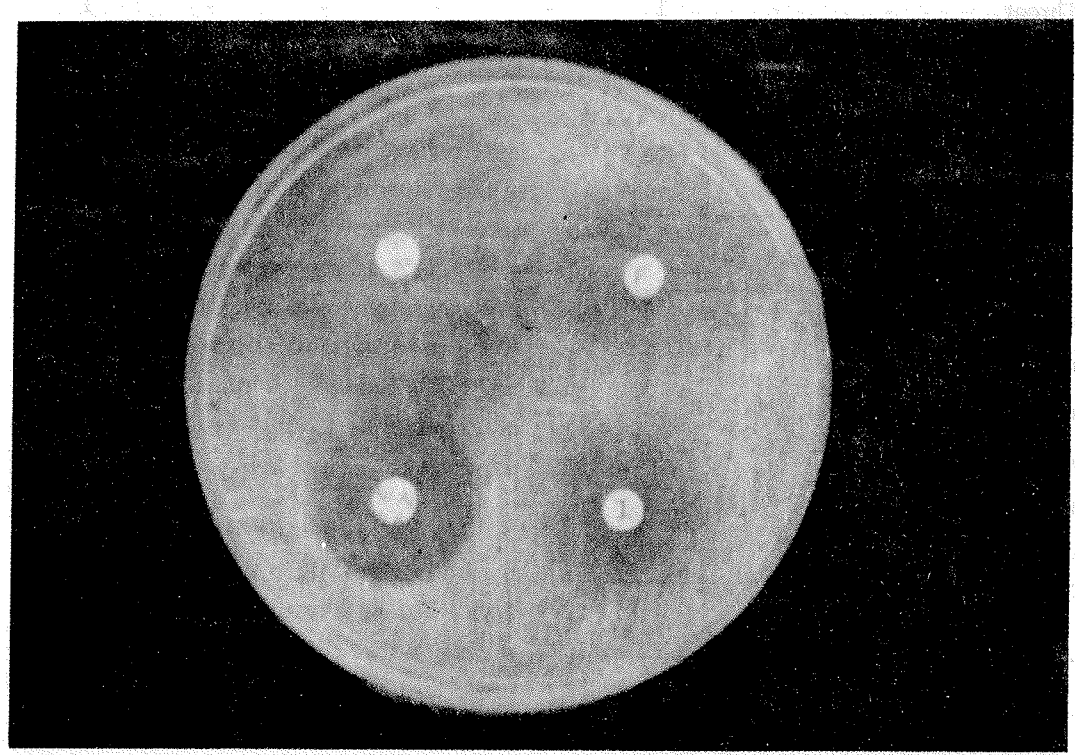

Fig. 1. Disc Sensitivity of Candida albicans to Nystatin, Amphotericin B, Fluconazole & Itraconazole.

Table 3 : Distribution of Candida isolates according to Prevalence (January-December 1997)

Specimens	C. tropi-	C. stell calis	C. albi- atoidea	C.parap cans	C. guill- silosis	C. krusei ermondii	Geotri -chum	Total
Blood	34	5	3	4	1	2	-	49
Tissue	2	-	-	1	4	1	1	9
Neckline	11	1	2	1	2	-	-	17
Pus	-	1	3	-	-	-	-	4
Urine	12	3	3	1	-	2	-	21
Fluids	3	-	1	-	-	-	-	4
Sputum	14	5	9	-	-	-	-	28
Suction	9	-	8	-	-	-	-	17
Faeces	1	-	-	2	-	2	-	5
Throat	-	-	1	-	-	-	-	1
Total	86	15	30	9	7	7	1	155

Table 4. Resistance of candida species to Fluconazole & Itraconazole.

	Fluconazole		Itraconazole	
	R	MS	MS	R
C.tropicalis	2	-	3	1
C.krusei	2	-	4	1
C.albicans	1	-	1	-
C.stellatoidea	-	-	-	1
Geotrichum candidum	-	1	-	1
Total	5 (3.2%)	1	8	3 (1.9%)

DISCUSSION

Methods of evaluating the susceptibility of yeasts to antifungal agents have been the subject of numerous studies during the last decade. A standard reference method described by NCCLS[5] which is a macro broth dilution assay is both cumbersome and time consuming to be used by clinical laboratories. The E test[6] (AB Biodisk, Solna, Sweden) which is capable of giving reliable results is expensive.

Macro and micro broth dilution methods give MIC'S which are extremely good. But in a routine clinical laboratory setting, a disc diffusion method has some important advantages. Multiple drugs can be tested and it is similar to the Kirby Baurer disc diffusion methods used to test antibacterial agents. It is important

to choose an appropriate medium, which is easy to prepare and dispense. The Bacto yeast nitrogen base agar used here has ammonia or asparagine as nitrogen sources and a pH of 7. Differences in pH may affect zone size or alter the activity of the drug itself. The medium is prepared in phosphate buffer which prevents drastic lowering of pH during incubation.

The routine candida isolates from the clinical laboratory were subjected to sensitivity testing. 155 clinical Candida strains isolated from blood, tissue urine, pus, sputum etc., were tested for sensitivity. All strains which showed resistant or moderately sensitive zones were retested twice and the average zone of inhibition was taken.

There were 86 C.tropicalis & 30 C.albicans isolates. The other species were C.stellatoidea (15), C.parapsilosis (9), C.guillermondii (7), C.krusei (7) and one species of Geotrichum candidum. In our hospital C.tropicalis was the predominant organism isolated from different clinical specimens. C.albicans is known to be the most common species isolated in different clinical specimens. C.albicans is known to be the most common species isolated in different studies. Perera et al reports on 40 isolations from 432 high vaginal swab cultures and C.albicans was the predominant species isolated forming 76% of the isolates.

All strains tested were found to be sensitive to Nystatin and Amphotericin B. Both these are polyenes, one a topical agent and the other an intravenous drug. Polyene drugs form complexes with ergosterol (the major sterol in fungal membranes) which opens channels in the membrane and causes leakage of critical intracellular constituents and causes subsequent cell death. Law et al (1994)[8] have reported that 96% of their isolates were sensitive to Amphotericin B and the rest were resistant to it.

Out of 155 strains tested by disc diffusion method in our study, five (3.2%) were resistant to Fluconazole, one (0.64%) was moderately sensitive and 149 (96.12%) of the strains were sensitive to Fluconazole. Resistance to Fluconazole has been reported to be increasing with 17.5%[8] to 11.8%[9] being reported in different studies. Resistance to Fluconazole is seen to be prevalent among patients with AIDS who have been treated with Fluconazole orally for prolonged periods.[8] Among our isolates two strains of *C.krusei*, two of *C.tropicalis* and one of *C.albicans* were found to be resistant to Fluconazole. One strain of Geotrichum candidum was moderately sensitive to Fluconazole.

Itraconazole[10], a newer triazole was also tested for sensitivity to all the Candida isolates. Four strains of C.krusei three of C.tropicalis and one of C.albicans showed moderate sensitivity to the drug.

Three strains (one each of Geotrichum, *C. tropicalis* & *C. stellatoidea*) were resistant to Itraconazole.

C.krusei is known for its relative resistance to azoles. Out of the seven isolates we had, two were found to be resistant to Fluconazole and four were moderatety sensitive to Itraconazole. Metzger et al[9] have also reported on four strains of *C.krusei* showing resistance to Fluconazole. Agar diffusion method[11,12] has been used by different authors for testing sensitivity of Candida isolates and Yeast nitrogen base medium has been found to be easy to prepare and easy to use in a routine clinical laboratory.

As a general recommendation, no topical or systemic antimycotic therapy should be started without reliable sensitivity tests and fungal reisolates during therapy should be tested for increasing resistance to the drug. As incidence of serious infections due to yeasts continue to escalate and reports of inherent or acquired resistance to antifungal agents emerge, invitro antifungal susceptibility testing of clinical isolates assumes increasingly significant role in over all therapeutic decision making.

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